



TITLE:

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The Use of Preserved Hematopoietic Tissues for Treatment of Mice Lethally Irradiated with Gamma Rays under High Dose Rate. (II)

Effect of Preserved Homologous Bone Marrow

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Mice were irradiated lethally with gamma rays under high dose rate and were treated with preserved homologous bone marrow. The homologous bone marrow was preserved in 15 % glycerol-Tyrodé's solution or 10 % dimethyl-sulphoxide TC 199 at -80°C as long as 360 days. 5×10^6 and 10×10^6 homologous bone marrow cells suspended in 15 % glycerol-Tyrodé's solution and preserved for 90 and 360 days, respectively, retained their ability to protect lethally irradiated mice from death. 10×10^6 homologous bone marrow cells suspended in 10 % DMSO-TC 199 retained the ability when the period of preservation was less than 270 days.

INTRODUCTION

Since Lorenz¹⁾ many workers reported the successful survival of lethally irradiated animals treated with hematopoietic tissues. However, Barnes and Loutit²⁾ reported a difference between the survivals in the mice treated with isologous bone marrow cells and in those treated with homologous bone marrow cells. The survival rate in the mice treated with homologous bone marrow (HBM) decreased after 21 or 30 days. In 1949, Polge, Smith and Parkes³⁾ reported a method of freezing and thawing of living cell suspension using as a protective medium. And then many workers⁴⁻¹²⁾ reported that the bone marrow cells preserved at low temperature using glycerol as a protective could improve the survival of lethally irradiated mice. Also, dimethyl-sulphoxide (DMSO) was ascertained by many workers¹³⁾¹⁴⁾ to protect living cells against freezing injury.

The following experiments were undertaken to ascertain that HBM cells which were suspended in 15 % glycerol-Tyrodé's solution or 10 % DMSO-TC 199 and preserved at -80°C as long as 360 days could protect lethally irradiated mice and the results were discussed

MATERIALS AND METHODS

Mice. Dd/s and Na₂ strain mice supplied exclusively from the Kyoto University Animals Center were used as recipients and donors, respectively. They

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were 8 to 9 week old femal mice weighing 20~25 g at the time of experiments.

Bone marrow suspension. Bone marrow obtained from bilateral femurs and tibias was suspended in 15 % glycerol-Tyrode's solution^{4~12)} or 10 % DMSO-TC 199 solution^{13~18)}. A standard technique for this procedure was described¹⁶⁾. HBM cells were injected via the tail vein of the recipients within 4 to 6 hours after irradiation.

Freezing and thawing. Slow freezing^{10,12,21,21~23)} means that the temperature was lowered at a rate of 1~2°C/min. from 0°C until -25°C and then cooled to -80°C at a rate not exceeding 10°C/min. The method of slow freezing and fast thawing was described in the previous paper¹⁶⁾.

Cell viability. The eosin-uptake test was performed as a screening test in the experiments. The method was described in the previous paper¹⁶⁾.

Irradiation. A Co⁶⁰ gamma-irradiation facility which belongs to the Institute for Chemical Research of Kyoto University was used in the experiments. This facility was described in detail by Okamoto and Nakayama²⁴⁾ and Shimizu *et al*²⁵⁾. All recipients were exposed to a single 900 r total body irradiation which resulted in LD₅₀/14 days and LD₁₀₀/21 days. The irradiated mice, after the bone marrow transplantation or injection of only Tyrode's solution, were kept in wooden boxes, each measuring 15×21×30 cm. Eight to ten mice in a box were fed wheat supplemented with dried fish every other day and were given tap water *ad libitum*.

RESULTS

1. Some Observations on Treatments with HBM Preserved for Short Periods at 0~4°C (Refrigerator)

Experiment 1. This experiment was carried out to protect lethally irradiated

Table 1. Survival rate of gamma-irradiated mice treated with preserved HBM.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival Number of irradiated (at days)						% Survival (at days)				
			14	21	30	60	90	120	21	30	60	90	120
Control	45	—	2/162	0/162	—	—	—	—	0	—	—	—	—
Fresh	4	10	21/31	18/31	17/30	9/30	6/30	5/30	58	56	30	20	16
2 hours	2	15	11/14	9/14	4/14	4/14	2/14	1/14	64	28	28	14	7
4 hours	2	15	10/15	8/15	6/15	3/15	2/15	2/15	53	40	20	13	13
2 days	3	20	15/21	9/21	7/21	4/21	1/21	1/21	42	33	18	4	4
4 days	2	26	14/16	8/16	8/16	8/16	8/16	8/16	50	50	50	50	50
7 days	3	36	13/19	7/19	6/19	6/19	6/19	6/19	36	31	31	31	31
14 days	2	99	0/15	—	—	—	—	—	—	—	—	—	—

The number of nucleated cells injected was 10 million.

They were suspended in Tyrode's solution and preserved at 0~4°C for 2 hrs, 4 hrs, 2, 4, 7 and 14 days.

Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

Fresh means the group of gamma-irradiated treated with fresh homologous bone marrow.

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mice with the HBM cells which were suspended in Tyrode's solution and preserved at 0~4°C (refrigerator) for short periods. The HBM cells were preserved for 2 hours, 4 hours, 4, 7 and 14 days. The survival rates in the experiments were shown in Table 1. Death occurring within 14 days after irradiation was taken as owing to the radiation syndrome or failure of treatment. In these experiments the number of nucleated cells injected was 10×10^6 . In the experiments using fresh HBM, the survival rates were as follows: 67 % at 14 days, 58 % at 21 days, 56 % at 30 days, 30 % at 60 days, 20 % at 90 days and 16 % at 120 days after irradiation. Therefore, 58 % survival rate at 21 days decreased to 16 % at 120 days after irradiation. In the experiments with a 2-hour preserved HBM, the survival rates were 64 % at 21 days and 7 % at 120 days. The survival rates in the experiments were 36 to 64 % at 21 days and 7 to 50 % at 120 days after irradiation. However, HBM which was preserved for 14 days did not protect the lethally irradiated mice. The survival rates in the mice treated with HBM decreased after 21 days.

Body weight changes. The body weight changes in the experiments with fresh, a 2-hour preserved HBM and a 2-day preserved HBM are shown in Fig. 1.

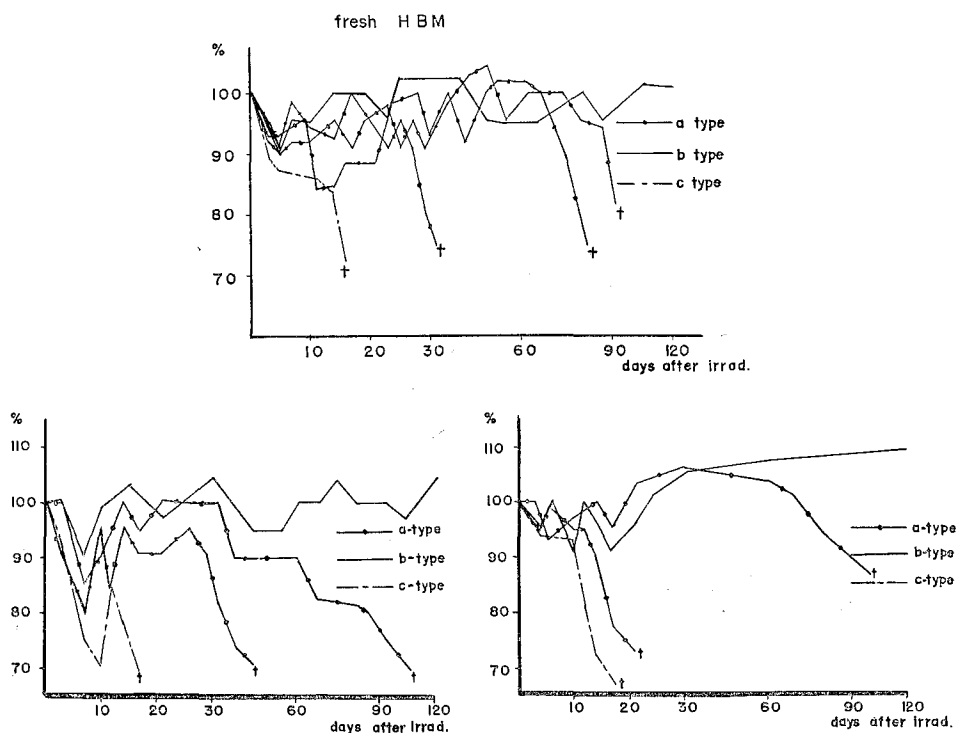


Fig. 1. Body weight changes in the mice irradiated lethally and treated with fresh, 2-hour and 2-day preserved homologous bone marrow. The number of nucleated cells injected was 10 million. They were suspended in Tyrode's solution.

The upper shows the changes in the fresh HBM treatment. The lower left shows the changes in the mice treated with HBM which was preserved for 2 hours at 0~4°C. The lower right shows the changes of body weight in the mice treated with HBM which was preserved for 2 days at 0~4°C.

Three types^{26,27)} of body weight changes were observed. a-type : This Type was seen in the mice which died between 21 days and 120 days after irradiation. Mice showed a rather slight degree of weight loss followed by a tendency to increase by the 20th day. Then the body weight started to decrease rapidly or gradually and the mice died. b-type : In this type, mice showed a moderate degree of weight loss followed by its gradually increase by the 30th day. However, some mice showed a temporary decrease after 30 days after irradiation. Some mice showed body weight changes as if they were treated with isologous bone marrow cells. The mice in this type survived for 120 days. c-type : In this type, mice showed a rapid and continuous decrease of body weight and died between 14 days and 20 days after irradiation. The death occurring after 21 days was considered as delayed death. Therefore, the mice, which died of delayed death, showed a-type in their body weight change.

Hematological findings. Leucocyte count. The changes of leucocyte count in the peripheral blood are shown in Fig. 2. Leucocyte count in the both controls and treated ones rapidly decreased to 500 to 1000 at 4 days after irradiation. In the controls there was never an increase in leucocyte count until the animals died. In the treated mice the leucocyte count began to increase at 5 to 10 days. Its recovery to the normal level was seen at 10 to 25 days after irradiation. The complete recovery of the peripheral leucocyte count in the mice which were treated with 4- and 7-day preserved HBM took place later than in the mice treated with HBM preserved for short periods. The leucocyte count in the mice treated with 14-day preserved HBM did not recover to the preirradiation level. Reticulocyte count. The changes of reticulocyte count are shown in Fig. 3. The reticulocyte count in the controls decreased to zero by 2 days. The count in the treated ones decreased to zero by 4 to 5 days followed by its increase with reticulocyte crisis. The peak of the crisis appeared at 12 to 22 days. The recovery of reticulocyte count in the mice transplanted with 2-, 4- and 7-day preserved HBM appeared later than in the mice transplanted with

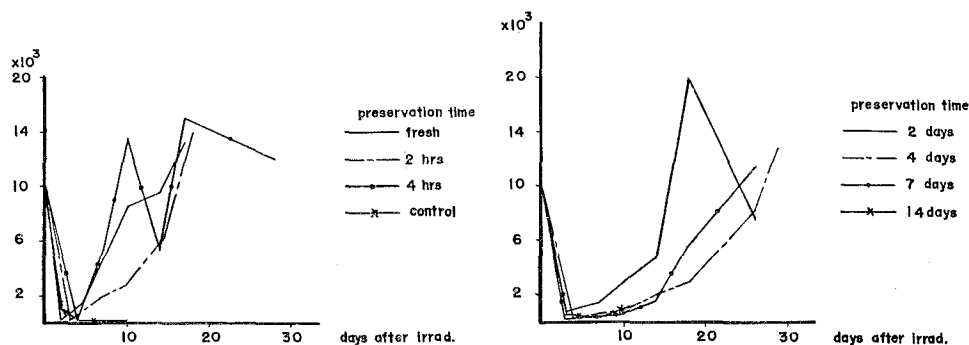


Fig. 2. Leucocyte count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in Tyrode's solution and preserved for 2 hrs, 4 hrs, 2, 4, 7 and 14 days at 0-4°C. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh HBM.

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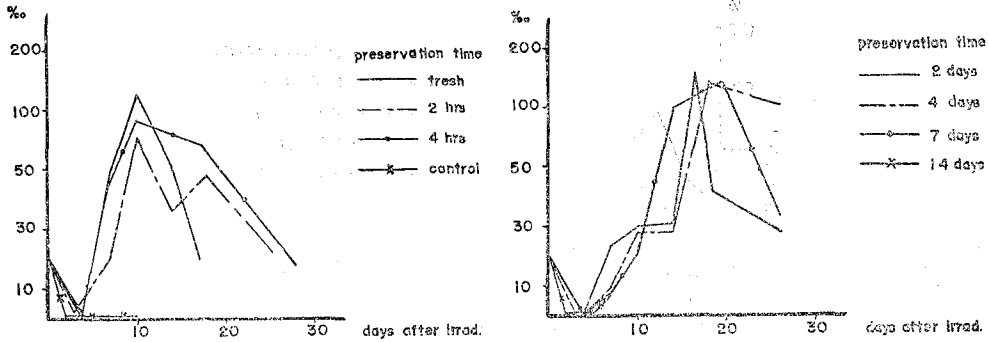


Fig. 3. Reticulocyte count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in Tyrode's solution and preserved for 2 hrs, 4 hrs, 2, 4, 7 and 14 days at 0-4°C. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh HBM.

HBM preserved for shorter periods. The mice transplanted with a 14-day-preserved HBM showed no recovery.

2. Some Observations on Treatment with HBM Preserved for Long Term at -80°C

Exp. 1. The experiment was carried out to protect the lethally irradiated mice with HBM cells which were preserved at -80°C as long as 180 days. The best method to preserve bone marrow cell suspension was slow freezing and fast thawing. The bone marrow cells were suspended in 15 % glycerol-Tyrode's solution and stored as long as 180 days. The number of nucleated cells injected was 5 million.

Table 2. Survival rate of gamma-irradiated mice treated with preserved HBM.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival (at days)					% Survival (at days)			
			Number of irradiated								
			14	21	30	60	90	21	30	60	90
Control	44	—	2/162	0/162	—	—	—	0	—	—	—
Fresh	5	10	27/65	12/65	11/65	6/60	3/60	18	17	10	5
30 days	2	13	8/16	5/16	3/16	2/16	2/16	31	18	12	12
60 days	4	17	18/25	11/25	8/25	6/25	5/25	44	32	24	20
90 days	2	25	6/10	5/10	4/10	2/10	2/10	50	40	20	20
120 days	2	26	1/13	0/13	—	—	—	0	—	—	—
150 days	1	33	0/8	—	—	—	—	0	—	—	—
180 days	2	51	3/14	0/14	—	—	—	0	—	—	—

The number of nucleated cells injected was 5 million.

They were suspended in 15 % glycerol-Tyrode's solution and preserved at -80°C for 30 to 180 days.

Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

Fresh means the group of gamma-irradiated mice treated with fresh homologous bone marrow.

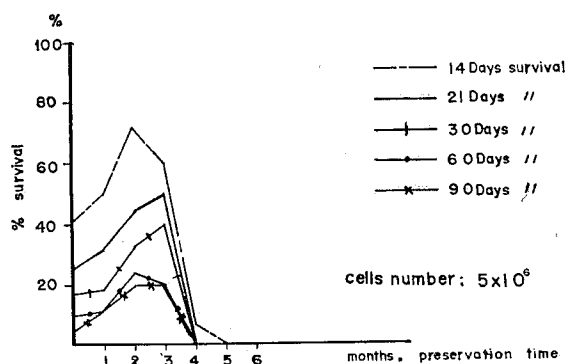


Fig. 4. Changes of percent survival in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 5 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 180 days at -80°C .

Survival rates. The survival rates are shown in Table 2 and Fig. 4. The survival rates in the experiment with fresh HBM were as follows: 41 % at 14 days, 18 % at 21 days, 17 % at 30 days, 10 % at 60 days, and 5 % at 90 days

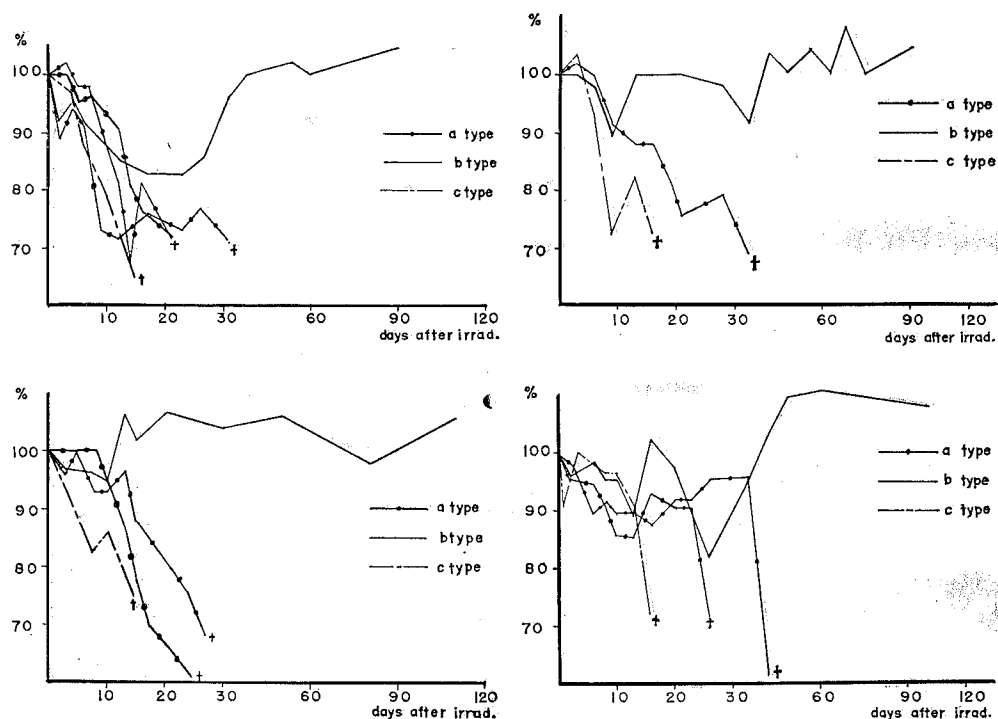


Fig. 5. Body weight changes in the mice irradiated lethally and transplanted with preserved HBM. The number of nucleated cells injected was 5 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 180 days at -80°C .

The upper left shows the changes in the fresh HBM treatment.

The lower left shows the changes in the 30-day preserved HBM treatment.

The upper right shows the changes in the 60-day preserved HBM treatment.

The lower right shows the changes in the 90-day preserved HBM treatment.

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after irradiation. As indicated in Table 2, the survival rate was 0 % at 21 days in the mice treated with HBM cells which were preserved for 120 days at -80°C . In the mice treated with 30- to 90-day preserved HBM, the survival rates were as follows: 41 to 72 % at 14 days, 18 to 50 % at 21 days, 17 to 40 % at 30 days, 10 to 24 % at 60 days and 5 to 20 % at 90 days after irradiation. The 90-day survival rates in the mice treated with 60- and 90-day preserved HBM were better than those in other preservation periods.

Body weight changes. The body weight changes in the mice treated with HBM which was fresh or preserved for 30 to 90 days are shown in Fig. 5. Three types described in Part 1 were recognized in this experiment. The c-type was recognized in the mice treated with HBM which was preserved for longer than 120 days.

Hematological findings. Leucocyte count. The changes of leucocyte count in the peripheral blood are shown in Fig. 6. The leucocyte count in the treated ones decreased rapidly to 500 to 1000 at 4 days. In the mice transplanted with 180-day preserved HBM, there was a temporary increase followed by a rapid decrease until they died. In the mice treated otherwise, the leucocyte count began to increase at 4 to 10 days. It approached gradually or quickly to the normal level and the time of recovery to the normal level was at 21 to 35 days after irradiation. The time of recovery to the normal level in the mice treated with preserved HBM came later than in those treated with fresh HBM.

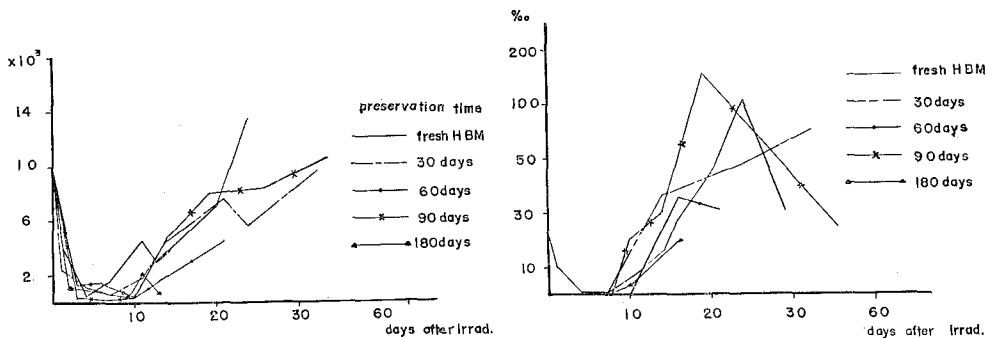


Fig. 6. Leucocyte count and reticulocyte count in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 5 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30, 60, 90 and 180 days at -80°C .

The right figure shows changes of leucocyte count in the treated mice.

The left figure shows changes of reticulocyte count in the treated mice.

Reticulocyte count. The changes of reticulocyte count in the peripheral blood are shown in Fig. 6. The reticulocyte count in the treated mice decreased to zero by 4 to 5 days and increased at 6 to 10 days. The reticulocyte count in the treated with 120- to 180-day preserved HBM showed no increase until the time of death. The mice treated with HBM preserved otherwise showed one reticulocyte crisis. The peaks of reticulocyte count appeared at 16 to 25 days. The reticulocyte count recovered to the preirradiation level at 25 to 40 days.

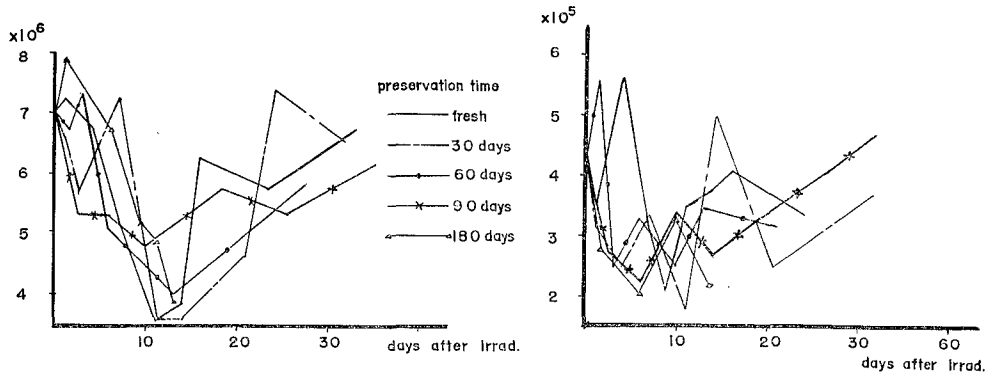


Fig. 7. Erythrocyte count and platelet count in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 5 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30, 60, 90 and 180 days at -80°C . Fresh means the group of gamma-irradiated mice treated with fresh HBM.

The left figure shows changes of erythrocyte count in the treated mice.

The right figure shows changes of platelet count in the treated mice.

Erythrocyte count. The changes of erythrocyte count in the peripheral blood are shown in Fig. 7. There were no apparent differences between the treated ones and the control by the 14th days. A temporary increase in erythrocyte count occurring in the treated mice was considered as a result to dehydration. The lowest level of the erythrocyte count occurred at 10 to 14 days. The erythrocyte count in the mice treated with 120- to 180-day preserved HBM continued to decrease until the time of death after a temporary increase.

Platelet count. The changes of platelet count in the peripheral blood are shown in Fig. 7. The platelet count in the mice treated with 120- to 180-day preserved HBM increased temporarily and then decreased quickly to the level of 220×10^3 until the time of death. In the transplantation of HBM preserved for different periods, the platelet count decreased to the level of 190 to 200×10^3 at 9 to 10 days and then increased rapidly or gradually to the level of preirradiation at 18 to 40 days. However, there were some cases which had two decreasing phases in the changes in platelet count. The second decreasing phase of the platelet count appeared at 10 to 21 days in the mice treated with 30- 60- and 90-day preserved HBM.

Histological findings. The examined tissues were the femoral and sternal bone marrow, the thymus, the mesenteric and brachial lymphnode, and the spleen. Bone marrow. In the treated mice early regeneration was initiated on the 4th to 7th day. In the mice treated with fresh to 90-day preserved HBM, the cellularity increased to prevent acute radiation death and recovered to a normal cellularity by the 21st day after irradiation. But, in the mice treated with 120- to 180-day preserved HBM, the bone marrow stayed hypocellular until the time of their death. The time of appearance of early regeneration and complete recovery was almost the same as described in the mice treated with the isologous bone marrow which was preserved for corresponding periods.

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Table 3. The time of appearance of early regeneration and complete recovery in the hematopoietic tissues in gamma-irradiated mice treated with preserved HBM.

Preservation period	Bone marrow		Spleen				Thymus		Lymphnode	
	eg.	cp.	red eg.	pulp cp.	white eg.	pulp cp.	eg.	cp.	eg.	cp.
Fresh	4	21	7	21	30	90	9	a	30	90
30 days	4	21	9	30	31	w	14	a	21	w
60 days	7	21	11	21	24	w	7	a	21	w
90 days	4	21	6	21	45	70-90	9	a	21	w
150 days	7	—	10	—	—	—	13	—	—	—

The number of nucleated cells injected was 5 million.

They were suspended in 15% glycerol-Tyrode's solution and preserved at -80°C for 30 to 180 days. Fresh means the group of gamma-irradiated mice treated with fresh homologous bone marrow.

eg. means early regeneration in the tissues in the mice which were lethally irradiated and treated with fresh and preserved HBM.

cp. means complete recovery in the tissues in the mice.

"a" means that the examined tissues were atrophic on the 90th day after irradiation.

"w" means that the tissues were wasted on the 90th day after irradiation.

Spleen. The red pulp. In the mice treated with fresh to 90-day preserved HBM, early regeneration was discernible on the 5th to 10th day and complete recovery occurred by the 21st day. In the mice treated with 120- to 180-day preserved HBM, the cellularity did not recover to the preirradiation level.

The white pulp. In the mice treated with fresh HBM, the white pulp on the 14th day consisted of reticular cells and a small number of plasma cells. Early regeneration was seen on the 30th day and complete recover was seen on the 90th day. In the mice treated with 30- to 90-day preserved HBM, early regeneration was seen by the 90th day. In the mice treated with 120- to 180-day preserved HBM, early regeneration was never seen.

Thymus. In the mice treated with fresh HBM, thymocytes in the thymic cortex were seen on the 9th day. However, the thymic medulla stayed wasted on the 14th day. The cellularity recovered to that of a normal thymus on the 90th day, though the thymus still looked small. In the mice treated with 120- to 180-day preserved HBM, thymocytes were discernible on the 7th to 13th day. However, the thymus stayed atrophic and complete regeneration was never seen by the time of their death.

Lymphnode. In the mice treated with fresh HBM, the lymphnode on the 30th day showed masses of lymphocytes and complete recovery was seen on the 90th day. In the mice treated with 120- to 180-day preserved HBM, the lymphnodes were completely wasted or full of plasma cells and reticular cells by the time of their death. The mice treated with HBM showed retarded regeneration or failure of regeneration in the lymphatic tissues²⁹⁾ as compared with isologous bone marrow treatment.

Exp. 2. This experiment was carried out to protect the lethally irradiated mice with 10×10^6 nucleated cells of HBM which was preserved up to 360 days.

The HBM cells were suspended in 15 % glycerol-Tyrodé's solution and were frozen slowly, stored and used after fast thawing.

Survival rates. The survival rates in the experiment are shown in Table 4 and Fig. 8. In the mice treated with fresh HBM, the survival rates were as follows: 67 % at 14 days, 58 % at 21 days, 56 % at 30 days, 33 % at 60 days, 20 % at 90 days and 16 % at 120 days after irradiation. In the mice treated with 30- to 360-day preserved HBM, the survival rates were as follows: 25 to 88 % at 14 days, 0 to 71 % at 21 days, 0 to 40 % at 60 days, 0 to 28 % at 120 days after irradiation. Except for a 270-day preserved HBM, the survival rates

Table 4. Survival rate of gamma-irradiated mice treated with preserved HBM.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival (at days)						% Survival (at days)				
			Number of irradiated										
			14	21	30	60	90	120	21	30	60	90	120
Control	44	—	2/162	0/162	—	—	—	—	0	—	—	—	—
Fresh	5	10	21/31	18/31	17/31	9/31	6/30	5/30	58	56	33	20	16
30 days	3	13	17/20	7/20	4/20	1/20	1/20	1/20	35	20	5	5	5
60 days	1	17	6/7	5/7	4/7	2/7	2/7	2/7	71	57	28	28	28
90 days	2	25	12/14	5/14	4/14	1/14	1/14	1/14	35	28	7	7	7
120 days	2	26	12/15	9/15	7/15	6/15	4/15	4/15	60	46	40	26	26
150 days	3	45	9/26	1/26	1/26	1/26	1/26	1/26	3	3	3	3	3
180 days	2	51	7/14	2/14	2/14	2/14	2/14	2/14	14	14	14	14	14
270 days	2	56	4/14	0/14	—	—	—	—	0	—	—	—	—
360 days	2	78	8/16	2/16	1/16	1/16	1/16	1/16	12	6	6	6	6

The number of nucleated cells injected was 10 million.

They were suspended in 15 % glycerol-Tyrodé's solution and preserved at -80°C for 30 to 360 days.

Control means the group of gamma-irradiated mice treated with only Tyrodé's solution or nothing.

Fresh means the group of gamma-irradiated mice treated with fresh homologous bone marrow.

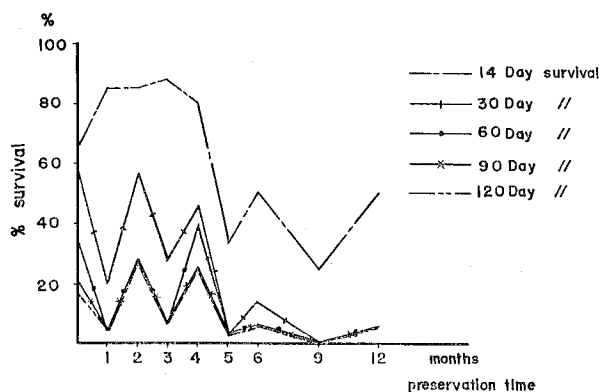


Fig. 8. Changes of percent survival in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 5 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C .

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were as follows: 33 to 88% at 14 days, 3 to 71% at 21 days, 3 to 57% at 30 days, 3 to 40% at 60 days, 3 to 28% at 90 days and 3 to 28% at 120 days. The best survival rates in the treated mice were obtained by the transplantation with 60- and 120-day preserved HBM.

Changes of body weight. Three types were seen as described in Part 1. c-type was not seen in 150-day preserved HBM treatment and only c-type was seen in 270-day preserved HBM treatment. The changes were shown in Fig. 9.

Hematological findings Leucocyte count. The changes of leucocyte count in the peripheral blood are shown in Fig. 10. In the treated mice, the leucocyte count in the peripheral blood decreased rapidly to 500~1000 at 3 to 5 days and then increased rapidly or gradually to the level of preirradiation, except in those treated with 270-day preserved HBM. The time of recovery to the preirradiation level was seen at 12 to 20 days in the mice treated with fresh to 90-day pre-

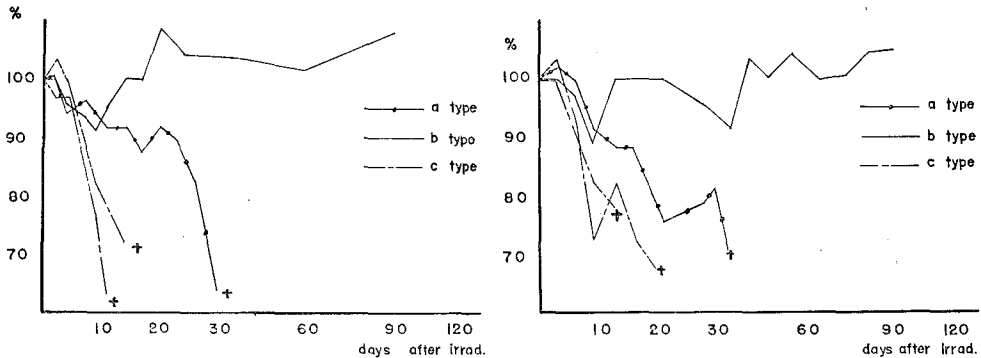


Fig. 9. Body weight changes in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved at -80°C .

The right shows body weight changes in the mice treated with 30 days preserved HBM.

The left shows the changes in the mice treated with 60 days preserved HBM.

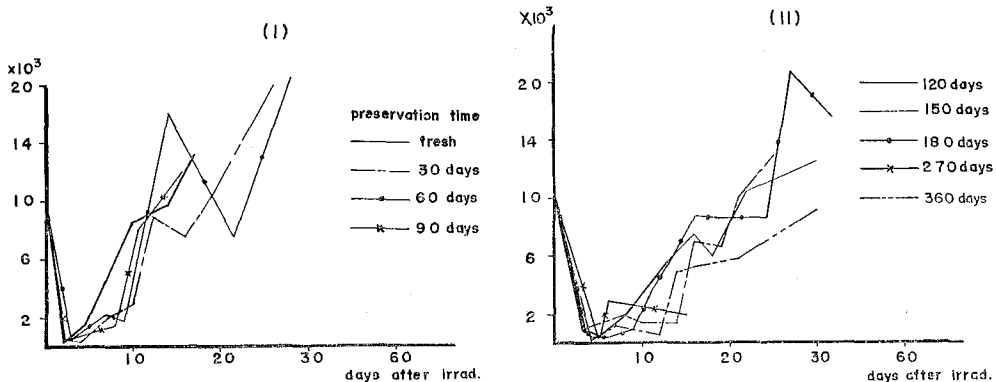


Fig. 10. Leucocyte count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C .

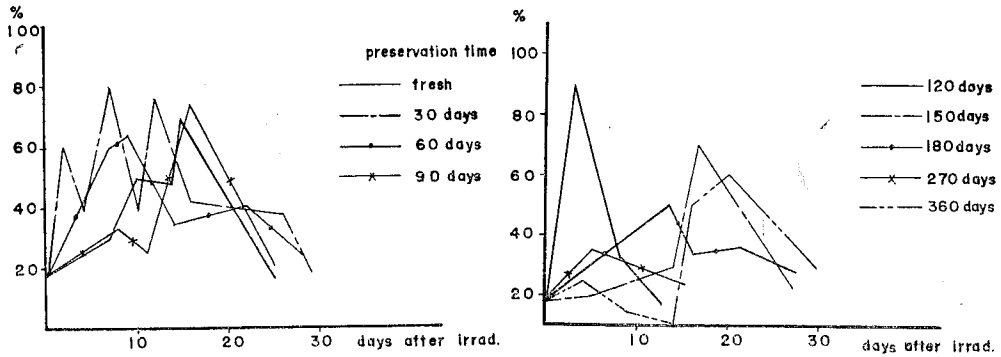


Fig. 11. Changes of percentage of neutrophils in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C .

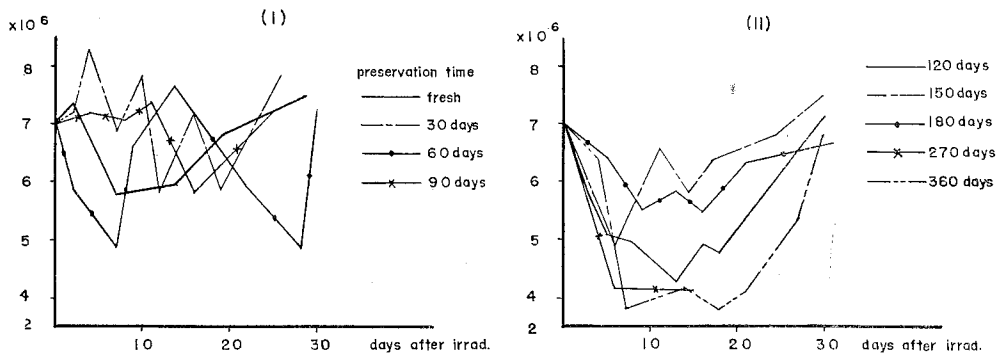


Fig. 12. Erythrocyte count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C .

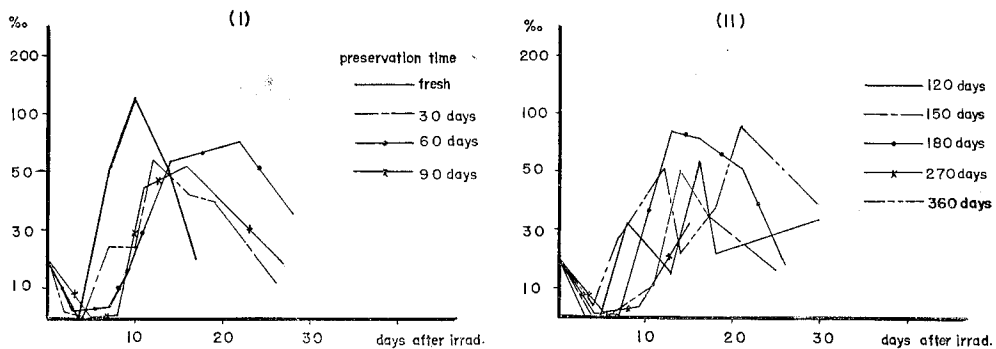


Fig. 13. Reticulocyte count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 15% glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C .

served HBM. However, recovery in the mice treated with 120-, 150-, 180-, and 360-day preserved HBM were seen at 20 to 30 days.

The percentage of neutrophils. In the peripheral blood of the mouse difference between lymphocyte and monocyte can not be made out clearly, so that both lymphocytes and monocytes are described as mononuclear cells¹⁰⁾. The changes of percentage of neutrophils are shown in Fig. 11. After treatment, the percentage increased quickly or gradually to 60 to 90 % from 18 % of preirradiation level and recovered to the preirradiation level by the 30th day after irradiation.

Erythrocyte count. The changes of erythrocyte count in the peripheral blood are shown in Fig. 12. After treatment, the erythrocyte count decreased quickly or gradually to 3×10^6 – 5.8×10^6 at 13 to 18 days and recovered to the preirradiation level at 25 to 30 days.

Reticulocyte count. The changes of reticulocyte count in the peripheral blood are shown in Fig. 13. After treatment, the reticulocyte count decreased to 0.2 % or less at 4 to 5 days and then increased to the peak of crisis at 10 to 21 days. In the mice treated with a 360-day preserved HBM, two peaks of the crisis were seen, one at 11 days and the other at 21 days. These results described above were not seen in the mice treated with a 270-day preserved HBM

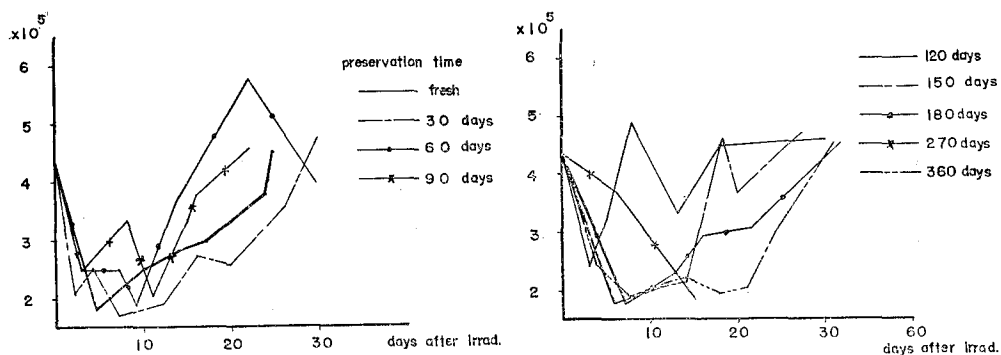


Fig. 14. Platelet count in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million.

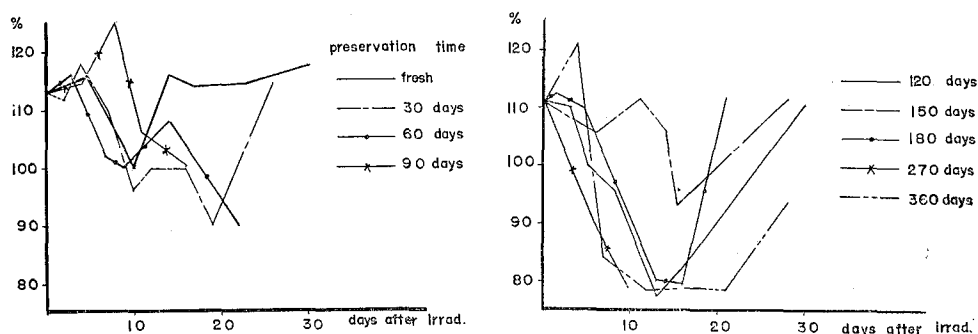


Fig. 15. Hemoglobin content in gamma-irradiated mice treated with preserved HBM. The number of nucleated cells injected was 10 million.

treatment.

Platelet count. The changes of platelet count in the peripheral blood are shown in Fig. 14. After treatment, platelet count decreased to 100×10^3 to 250×10^3 at 2 to 8 days and then recovered to the level of preirradiation at 16 to 28 days.

Hemoglobin content. Hemoglobin content in the peripheral blood was examined by the Sahli method. The changes of hemoglobin content in the treated mice are shown in Fig. 15. The hemoglobin content decreased to the level of 78 to 100 % at 10 to 18 days and then increased to the level of preirradiation at 12 to 30 days.

Histological findings. Bone marrow. In the treated mice early regeneration occurred between the 4th and 7th day and the time of recovery to a normal cellularity was between the 12th and 21st day, except in those treated with a 270-day preserved HBM.

Table 5. The time of appearance of early regeneration and complete recovery in the hematopoietic tissues in gamma-irradiated mice treated with preserved HBM.

Preservation period	Bone marrow		Spleen				Thymus		Lymphnode	
	eg.	cp.	red eg.	pulp cp.	white eg.	pulp cp.	eg.	cp.	eg.	cp.
Fresh	4	14	5	21	40	90	—	90	30	90
60 days	4	16	7	21	—	100	—	90	—	90
120 days	5	18	6	25	60	100	—	96	—	96
150 days	5	14	7	20	—	120	—	120	—	100
180 days	5	20	5	21	—	120	—	120	—	120
270 days	5	—	7	—	—	—	—	—	—	—
360 days	5	21	8	21	—	100	—	100	—	100

The number of nucleated cells injected was 10 million.

They were suspended in 15 % glycerol-Tyrodé's solution and preserved at -80°C for 30 to 360 days.

Fresh means the group of gamma-irradiated mice treated with fresh homologous bone marrow.

eg. means early regeneration in the tissues in the mice which were lethally irradiated and treated with fresh and preserved HBM.

cp. means complete recovery in the tissues of the mice.

Spleen. In the mice treated with fresh to a 360-day preserved HBM, early regeneration in the red pulp was discernible on the 6th to 10th day. In the treated mice, except in those treated with a 270-day preserved HBM, complete recovery in the red pulp occurred between the 12th to 21st day. Also in the treated mice, recovery of the white pulp began no earlier than the 40th day. Complete recovery of it occurred between the 90th and 120th day.

Thymus. After treatment, the thymus stayed atrophic and had a small number of thymocytes in the cortex in some experiments. Complete recovery in the fresh HBM transplantation was seen on the 41st day. In other experiments, complete recoveries were never seen before the 90th or the 120th day after irradiation.

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Lymphnode. The lymphnode stayed wasted and were full of reticular cells and plasma cells. Complete recovery was never seen before the 90th to 120th day.

Exp. 3. This experiment was carried out to protect the lethally irradiated mice with HBM cells which were kept at -80°C for 30 to 360 days. The HBM cells were suspended in 10 % dimethyl-sulphoxide (DMSO) TC 199 and were frozen slowly, stored and used after fast thawing. The number or nucleated cells injected was 10 million.

Survival rates. The survival rates in the experiment are shown in Table 6 and Fig. 16. Except in the experiment with fresh and 360-day preserved HBM, the survival rates in other experiments were as follows : 44 to 73 % at 14 days, 5 to 60 % at 21 days, 5 to 33 % at 30 days, 5 to 26 % at 60 days, 5 to 20 % at 90 days and 5 to 13 % at 120 days. The survival rate in the mice treated with a 360-day preserved HBM were as follows : 11% at 14 days, 3% at

Table 6. Survival rate of gamma-irradiated mice treated with preserved HBM.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival (at days)						% Survival (at days)					
			Number of irradiated											
			14	21	30	60	90	120	21	30	60	90	120	
Control	44	—	2/162	0/162	—	—	—	—	0	—	—	—	—	—
Fresh	5	10	21/31	18/31	17/31	9/31	6/30	5/30	58	56	33	20	16	
30 days	2	12	6/14	3/14	1/14	1/14	1/14	1/14	21	7	7	7	7	
60 days	2	12	9/15	2/15	2/15	1/15	1/15	1/15	15	6	6	6	6	
90 days	2	22	7/16	6/16	4/16	1/16	1/16	1/16	37	25	6	6	6	
120 days	2	37	10/15	9/15	5/15	4/15	3/15	2/15	60	33	26	20	13	
150 days	2	41	8/18	1/18	1/18	1/18	1/18	1/18	5	5	5	5	5	
180 days	2	48	11/15	1/15	1/15	1/15	1/15	1/15	6	6	6	6	6	
270 days	2	50	11/15	6/15	5/15	2/15	2/15	1/15	40	33	13	13	6	
360 days	3	60	3/26	1/26	0/26	—	—	—	3	0	—	—	—	

The number of nucleated cells injected was 10 million. They were suspended in 10 % DMSO-TC 199 solution and preserved for 30 to 360 days at -80°C .

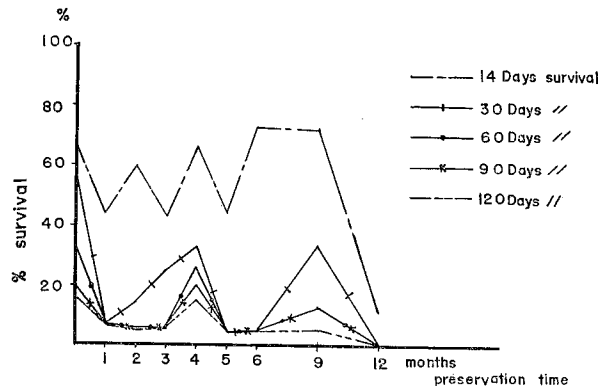


Fig. 16. Changes of percent survival in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 10% DMSO-TC199 solution and preserved for 30 to 360 days at -80°C .

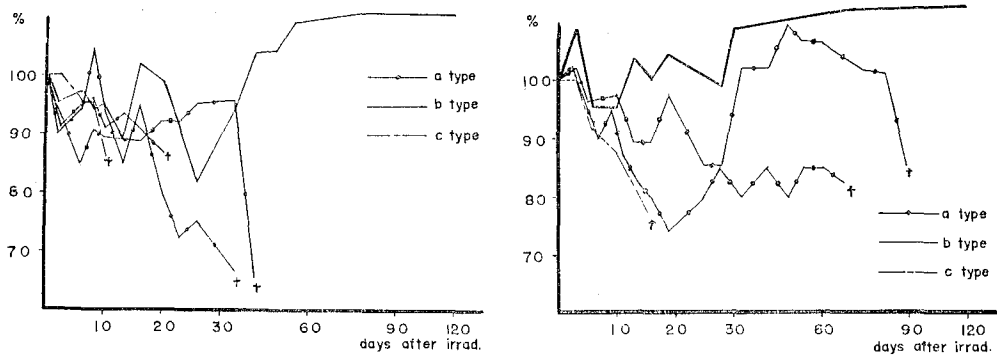


Fig. 17. Body weight changes in the mice irradiated lethally and treated with preserved HBM. The number of nucleated cells injected was 10 million. They were suspended in 10% DMSO-TC199 solution and preserved at -80°C . The right shows body weight changes in the mice treated with a 90-day preserved HBM. The left shows the changes in the mice treated with a 120-day preserved HBM.

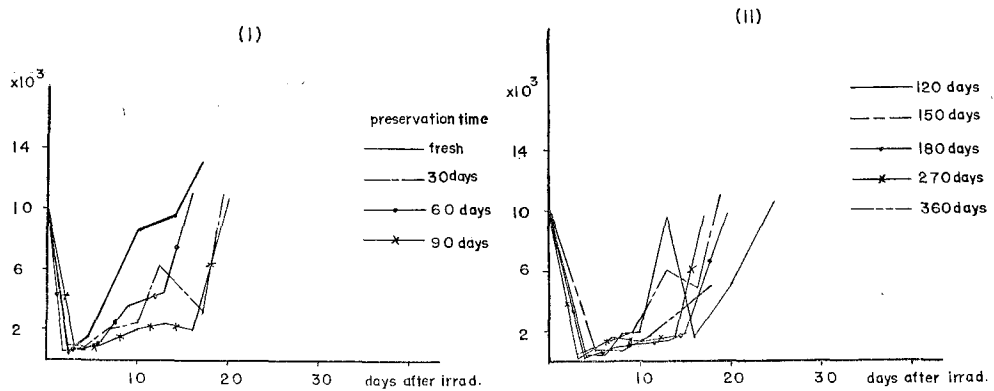


Fig. 18. Leucocyte count in gamma-irradiated mice treated with preserved HBM.

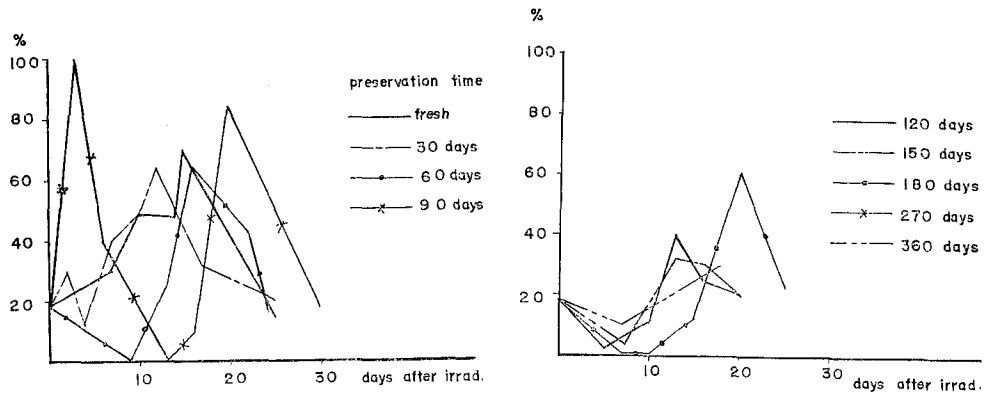


Fig. 19. Changes of percentage of neutrophils in gamma-irradiated mice treated with preserved HBM.

21 days and 0 % at 30 days after irradiation.

Changes of body weight. Three types consisting of a-, b-, and c-type were seen in the treated mice. However, the a-type was not seen in the mice treated with a 180-day preserved HBM and the b-type was not seen in the mice treated with a 360-day preserved HBM. The change are shown in Fig. 17.

Hematological findings. Leucocyte count. The changes of leucocyte count in the peripheral blood are shown in Fig. 18. In the treated mice, leucocyte count decreased rapidly to 500 at 3 to 7 days and then increased rapidly or gradually to the level of preirradiation. The time of recovery to the preirradiation level was at 13 to 20 days in the mice treated with 30- to 270-day preserved HBM, but it was never observed in the mice treated with 360-day preserved HBM.

The percentage of neutrophils. The changes of percentage of neutrophils are shown in Fig. 19. After treatment, the percentage increased quickly or gradually to 35 to 100 % from 18 % of preirradiation level and recovered to the preirradiation level by the 30th day.

Erythrocyte count. Changes of erythrocyte count in the peripheral blood are shown in Fig. 20. After treatment, the erythrocyte count decreased quickly or gradually to 4.3×10^6 to 5.7×10^6 at 14 to 20 days and recovered to the preir-

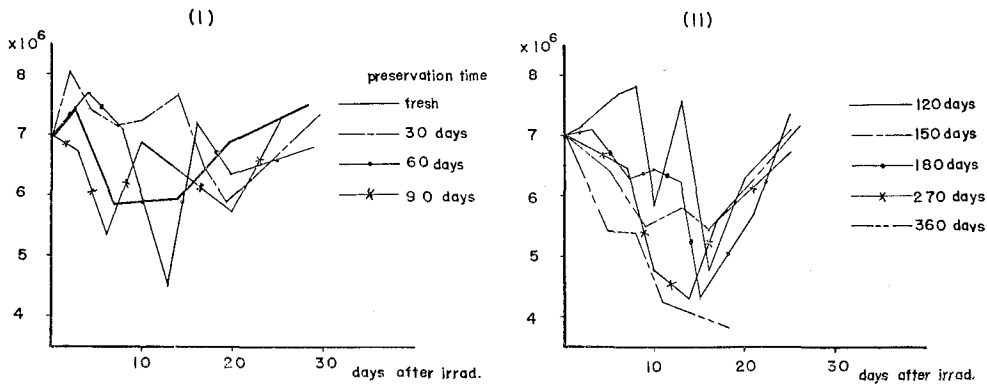


Fig. 20. Erythrocyte count in gamma-irradiated mice treated with preserved HBM.

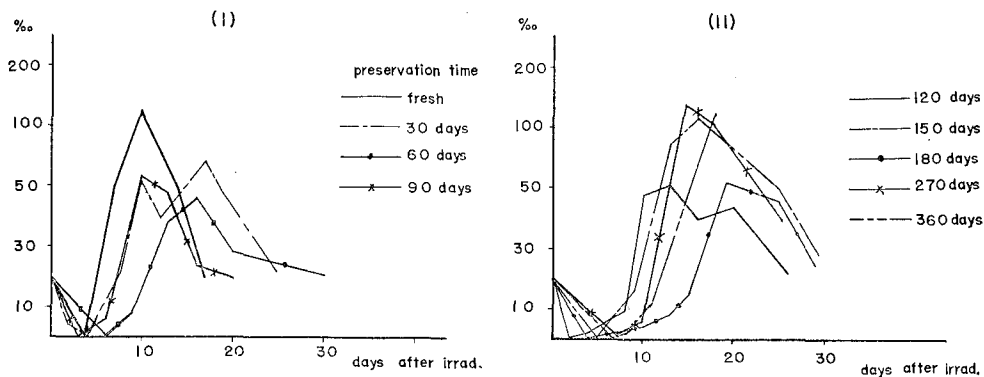


Fig. 21. Reticulocyte count in gamma-irradiated mice treated with preserved HBM.

radiation level at 25 to 30 days. However, the recovery in the mice treated with a 360-day preserved HBM was never seen.

Reticulocyte count. Changes of reticulocyte count in the treated mice were shown in Fig. 21. After treatment, the reticulocyte count decreased to 2 to 0% at 4 to 5 days and then increased to the peak of crisis at 10 to 21 days. In the mice treated with a 360-day preserved HBM, the peak was never seen.

Platelet count. Changes of platelet count in the treated ones are shown in Fig. 22. After treatment, platelet count decreased to 50×10^3 to 200×10^3 at 7 to 16 days and then recovered to the preirradiation level at 21 to 30 days after irradiation. However, the recovery was never seen in the mice treated with a 360-day preserved HBM.

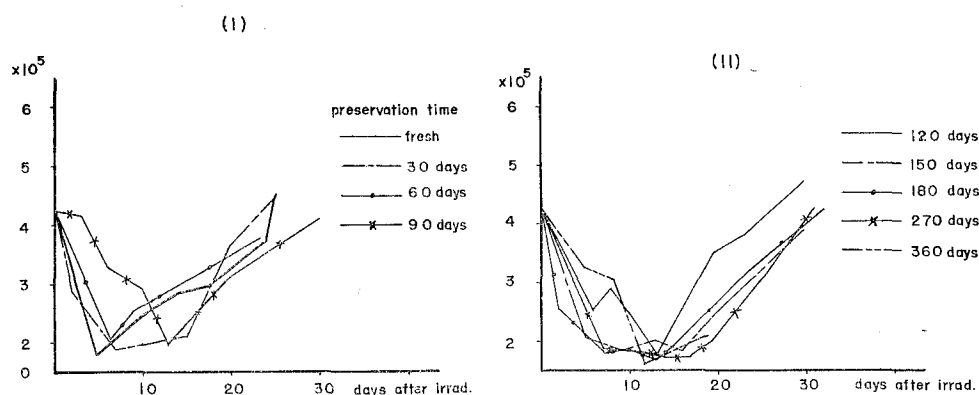


Fig. 22. Platelet count in gamma-irradiated mice treated with preserved HBM.

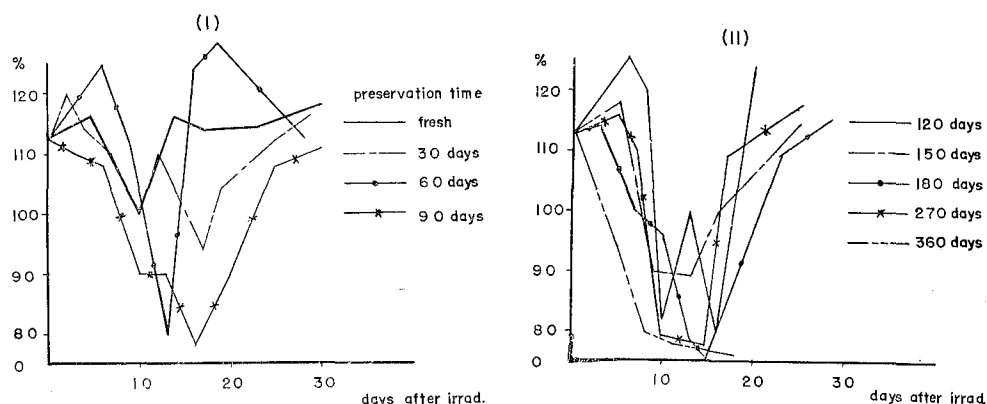


Fig. 23. Hemoglobin content in gamma-irradiated mice treated with preserved HBM.

Hemoglobin content. Changes of hemoglobin content are shown in Fig. 23. The hemoglobin content in the treated mice decreased to the level of 70 to 100% at 10 to 17 days and then increased and reached to the level of preirradiation at 12 to 28 days. However, in the mice treated with a 360-day preserved HBM, recovery was never seen.

Histological findings. The examined tissues were the same as those in the

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Table 7. The time of appearance of early regeneration and complete recovery in the hematopoietic tissues in gamma-irradiated mice treated with preserved HBM.

Preservation period	Bone marrow		Spleen				Thymus		Lymphnode	
	eg.	cp.	red eg.	pulp cp.	white eg.	pulp cp.	eg.	cp.	eg.	cp.
Fresh	4	14	5	21	40	90	—	90	30	90
60 days	4	14	8	21	—	90	—	90	—	90
120 days	5	14	7	21	—	100	—	100	—	100
180 days	4	14	7	21	—	120	—	120	—	120
270 days	4	15	6	21	—	120	—	120	—	120
360 days	4	14	7	21	—	—	—	—	—	—

The number of nucleated cells injected was 10 million.

They were suspended in 10 % DMSO-TC 199 solution and preserved at -80°C for 30 to 360 days. Fresh means the group of gamma-irradiated mice treated with fresh HBM.

eg. means early regeneration in the tissues in the mice which were lethally irradiated and treated with fresh and preserved HBM.

cp. means complete recovery in the tissues in the mice.

experiments described previously. The time of early regeneration and complete recovery are shown in Table 7. The times were almost the same as described in the treated mice in Exp. 2.

DISCUSSION

It is now firmly established that lethally irradiated mice can be protected from hematopoietic death by the intravenous injection of isologous or foreign hematopoietic tissues¹⁾ after irradiation. By treating the mice with isologous bone marrow, an excellent survival can be obtained. However, when irradiated mice are treated with homologous bone marrow, death occurs starting at 21 to 30 days after irradiation^{28~31)}. It has been shown that recovery is due to recolonization of the host's radiation-damaged hematopoietic tissues by the normal cells injected^{32~35)}. Therefore, living hematopoietic cells can protect lethally irradiated mice from death. The isologous bone marrow which was preserved for 270 to 360 days at -80°C could protect lethally irradiated mice from hematopoietic death as well as fresh isologous bone marrow did. Based on the results, the homologous bone marrow cells also were preserved for a long period.

The isologous bone marrow which was preserved at $0\sim4^{\circ}\text{C}$ for less than 3 days could protect irradiated mice from death. The homologous bone marrow was preserved for from 2 hours to 14 day at $0\sim4^{\circ}\text{C}$. The number of nucleated cells injected was 10 million. The 30-day survival rate in the mice treated with HBM which was preserved for less than 4 days were the same as that in the mice treated with fresh HBM. The 90-day survival rate better than that in the mice treated with fresh HBM was obtained with HBM which was preserved for 4 days and 7 days. As indicated in Table 1, delayed death after the 21st day occurred in the treated ones. As the characteristic findings of the

delayed mortality, the animals were emaciated and showed weight loss and extensive depilation and diarrhea occurred in some animals. The characteristic histological findings were retarded recovery or wasting of the lymphatic tissues.

To preserve the living cells, the first essential method is to reduce the rate at which physical and chemical phenomena proceed. This is obtained by a low temperature. The formation of ice from water is the most important physical changes occurring when the cells are carried to a low temperature. According to modern cryobiology^{22,23)}, the intracellular ice formation is fatal to the cells when they are frozen. To preserve the cells, it is essential to prevent ice formation inside the cells. When freezing is relatively slow, ice crystals appear to form exclusively in the extracellular space. With increasing rate of cooling, the tendency of extracellular crystal formation diminishes and ice crystals begin to appear inside the cells.

In 1949, Polge and coworkers³⁾ reported the first successful freezing of bovine semen using glycerol as a protective medium. And then dimethyl-sulphoxide and polyvinylpyrrolidone were found to be protective from freezing injury. The best protective compounds were found among either polyalcohols or monosaccharides. To preserve the cells for a long term, the cells must be frozen slowly in the presence of these compounds. Bender⁹⁾, Tran¹⁰⁾, and Ferrebee⁵⁾ reported that the best volume percent of glycerol was 15 % V/V for the preservation of living cells. Ashwood-Smith¹⁴⁾ reported that mouse bone marrow was prevented from injury of freezing with dimethyl-sulphoxide as the protective medium. By Ashwood-Smith¹⁴⁾ and Richards¹¹⁾ the best volume percent was 10 % V/V. Therefore, 15 % glycerol-Tyrodé's solution or 10 % DMSO-TC 199 solution was used for the mouse bone marrow preservation. The bone marrow suspension were frozen slowly, stored and used after fast thawing.

The HBM was preserved for 30 to 180 days at -80°C . The number of nucleated cells injected was 5 million. The HBM preserved for more than 120 days did not protect the lethally irradiated mice from acute radiation death. The 30-day survival rate in the mice treated with HBM which was preserved for 60 and 90 days was better than that in fresh and 30-day preserved HBM treatment. The 90-day survival rate better than fresh and 30-day preserved HBM treatment was obtained with HBM which was preserved for 60 and 90 days. The characteristic findings usually found in the delayed death were seen in the mice died.

The HBM was suspended in 15 % glycerol-Tyrodé's solution and preserved for 30 to 360 days at -80°C . The number of nucleated cells injected was 10 million. Generally speaking, the HBM which was preserved for not more than 120 days could protect the lethally irradiated mice from acute radiation death as well as the fresh HBM did. However, the 120 day survival rate in the mice transplanted with HBM preserved for longer than 150 days was inferior to that in the experiments using HBM preserved for shorter periods.

The HBM was suspended in 10 % DMSO-TC 199 solution and preserved for 30 to 360 days at -80°C . The number of nucleated cells injected was 10 million. Generally speaking, the preserved HBM did not protect the irradiated mice so

well as fresh HBM did.

As for the changes of body weight in the mice treated with HBM, three types were reported. According to Yamagishi¹³⁾, the three types of weight changes are as follows: 1. Mice which showed a rapid and continuous decrease of body weight. The mice died between 14 and 20 days. This type was described as the c-type in the experiment. 2. Mice which showed a moderate degree of weight loss followed by its gradual or rapid increase beginning around the 21st day. The mice did not die before the 120th day. In some mice showing this type, the change of body weight was approximately the same as that in the mice treated with the isologous bone marrow. This type was described as the b-type in the experiment. 3. Mice which showed a rather slight degree of weight loss followed by a tendency to increase by the 21st day. Then the body weight started to decrease gradually or rapidly and the mice died between 21 and 120 days. This type was described as the a-type in the experiment. According to Makinodan²⁷⁾, three types were recognized in the changes of body weight in the mice treated with rat bone marrow. However, c-type in our classification was not reported in his three types. A-type in this experiment contained two subtypes in his types. Only one or two types were seen in some experiments. C-type was not that of the characteristic delayed death, because irradiated mice without treatment died between 14 and 20 days. Typical delayed death was recognized as a-type in the experiment.

The hematological findings in the peripheral blood returned to the level of preirradiation before 30 to 40 days after irradiation. The recovery was recognized in the mice suffered from the secondary disease.

As for the histological findings in the experiments with HBM, two different recovery processes were seen. The bone marrow and the red pulp of spleen showed recovery from complete wasting to the level of preirradiation after irradiation before 21 to 28 days. The recovery process was approximately the same in the mice treated with isologous bone marrow. After treatment with HBM, complete regeneration of the thymus was observed on the 90th to 120th day. Regeneration of the lymphatic tissues (the lymphnode and the white pulp of spleen) was retarded or failed to occur in the mice treated with HBM. The time of complete recovery in the lymphatic tissues was shown in Table 5 which showed a retarded recovery as compared with isologous bone marrow transplantation. In a number of HBM-transplanted animals with or without secondary disease sacrificed for the histological examination on the 21st day, the lymphatic tissues consisted of reticular cells, devoid of lymphoid cells, and were fully infiltrated with plasma cells. Therefore, it was found that the histologically characteristic findings in the mice transplanted with HBM were delayed or incomplete recovery of the lymphatic tissues.

Two theories are proposed on the pathogenesis of the secondary disease and delayed death^{29-31, 36-41)}. These theories are based on the assumption that the death occurs as a result of immunological antigen-antibody reaction. The first theory is that the host-versus-graft reaction is postulated as the cause of death³⁹⁻⁴¹⁾. The other theory is that the secondary disease and delayed death

Explanations of Plates

Plate 1. Bone marrow; 4 days after 900r gamma-irradiation and treatment with fresh HBM. The number of nucleated cells injected was 5 million. A group of immature bone marrow cells are seen.

H-E stain $\times 400$

Plate 2. Bone Marrow; 15 days after 900r gamma-irradiation and treatment with 270-day preserved HBM suspended in 15% glycerol-Tyrode's solution. The cellularity is normal.

H-E stain $\times 100$

Plato 3. Brachial lymphnode; 63 days after 900r gamma-irradiation and treatment with 120-day preserved HBM suspended in 15% glycerol-Tyrode's solution. The lymphnode is wasted.

H-E stain $\times 400$

Plato 4. White pulp; 93 days after 900r gamma-irradiation and treatment with 120-day preserved HBM suspended in 15% glycerol-Tyrode's solution. A marked increase in number of lymphocytes in the white pulp.

H-E stain $\times 400$

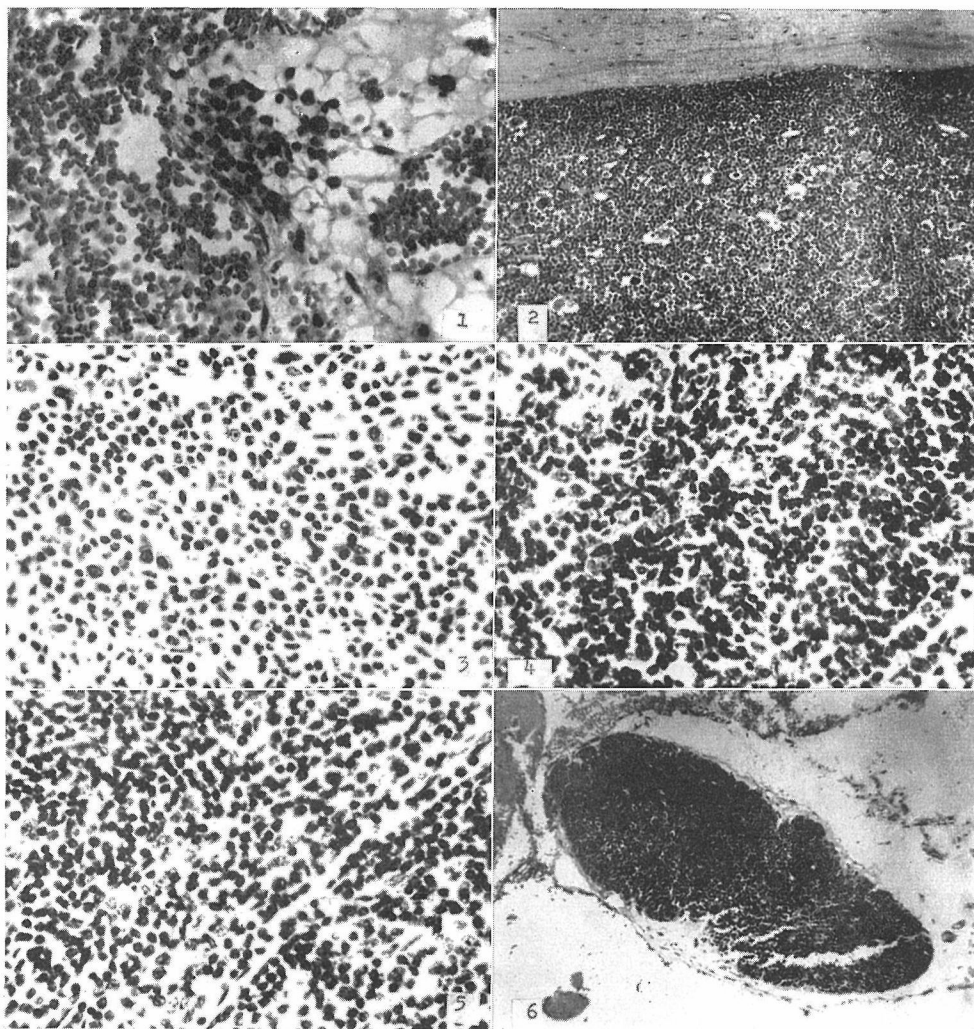
Plato 5. Brachial lymphnode; 96 days after 900r gamma-irradiation and treatment with 120-day preserved HBM suspended in 10% DMSO-TC199 solution. A little increase in number of lymphocytes in the lymphnode.

H-E stain $\times 400$

Plato 6. Thymus; 120 days after 900r gamma-irradiation and treatment with 120-day preserved HBM suspended in 10% DMSO-TC199 solution. The thymus stayed atrophic.

H-E stain $\times 100$

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after treatment with HBM are attributed to the graft-versus-host reaction^{29~31, 36~38)}. The factors influencing transplantation immunity are the dose of irradiation, age of the donor, number of donors cells, and genetic disparity between donor and recipient. Recent evidence has given much support to the graft-versus-host reaction. If there exists a graft-versus-host reaction when adult fresh bone marrow is used, what result can be obtained by the use of HBM which was preserved at a low temperature? There are immunologically competent and incompetent cells in the bone marrow.

The effect of storage at 0~4°C. In the author's experiments, the HBM which was prererved for 4 and 7 days at 0~4°C could protect the lethally irradiated mice better than fresh HBM. Therefore, the inactivating effect of storage for 4 and 7 days at 0~4°C was found to be more pronounced on immunologically competent cells than on the hemopoietic cells (the immunologically incompetent cells)⁴²⁾.

The effect of storage at -80°C⁴³⁾. In the author's experiments, when the HBM was preserved for long term at -80°C with glycerol as a protective additive and the number of nucleated cells injected was 5×10^6 , the better 90 day survival rate was obtained in the 30-, 60- and 90-day preserved HBM transplantation than in the fresh HBM transplantation. The immunologically incompetent cells were inactivated when the HBM was preserved for 120 days at -80°C. When the HBM was suspended in 15 % glycerol-Tyrodé's solution and stored for 360 days at -80°C and the number of nucleated cells injected was 10×10^6 , delayed death was not prevented. HBM preserved for 120 days or less protected acute radiation death as well as fresh HBM did, but not the HBM preserved for 150 days or more. The 120-day survival rates better than in fresh HBM transplantation, was obtained by 60- and 120-day preserved HBM transplantation. When the HBM was suspended in 10 % DMSO-TC 199 solution and stored for 360 days at -80°C and the number of nucleated cells injected was 10×10^6 , good protective results for acute radiation injury were obtained. However, the 120-day survival rate in the preserved HBM transplatation was inferior to that in the fresh HBM transplantation.

SUMMARY

1. The HBM preserved for 7 days or less at 0~4°C protected lethally irradiated mice. The HBM preserved for 4 and 7 days protected the irradiated mice better than fresh did.
2. The HBM suspended in 15 % glycerol-Tyrodé's solution and stored for 120 days or less protected the irradiated mice as well as fresh HBM did.
3. The HBM suspended in 10 % DMSO-TC 199 solution and stored for a long period did not protect the lethally irradiated mice so well as fresh HBM did.

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